Splinkerette PCR Protocol for Mapping Transposable Elements in *Drosophila*

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Protocol Outline

1) Genomic Prep (30 mins)
2) Genomic Digest (2 hrs → O/N)
3) Ligation of digested genomic DNA to annealed splinkerette oligonucleotide (2 hrs)
4) Round 1 PCR (2 hrs)
5) Round 2 PCR (1.5 hrs)

6a) Ant Phos/ExoI treat Round 2 DNA (2hrs)

6b) or Run Round 2 PCR on gel and purify band (1 hr)

7) Sequence

Step 1. Genomic Prep

There are many established methods for genomic DNA preparations. When performing splinkerette PCR on a small number of lines, we recommend the QIAGEN DNeasy kit (Qiagen Inc., Valencia, CA). Elute in 200 µl AE buffer. Typical DNA concentrations are ~ 20-50 ng/µl.

For many samples (*e.g.,* 96 well plates) follow the genomic preparation protocol established in [1]. The splinkerette protocol also works with a simple genomic prep from single flies (http://engels.genetics.wisc.edu/flyDNA.html).

Step 2. Genomic Digest

Table 1. Genomic Digest Reaction.

Components	Volume
genomic DNA (~20-40 ng/µl)	25 µl
H_2O	2 µl
10X BSA	3.5 µl
10X NEB BUFFER	3.5 µl
ENZYME	1 µl
Total	**35 µl**

Table 2. Restriction Enzyme Conditions (to generate GATC sticky ends).

Enzyme	Cut Site	Digest Temp	Heat inactivate?	Purify digest?
BamH1	G↓GATCC	37˚C	No	yes
BglII	A↓GATCT	37˚C	No	no
BstYI	R↓GATC Y	60˚C	Yes, 20 min @80˚C	no
BfuCI	↓GATC	37˚C	Yes, 20 min @80˚C	no

2.1 See Table 12 for details on the compatible restriction enzymes. Digest genomic DNA ≥ 2 hrs. As listed in Table 2, heat inactivate or purify the enzymatic reaction if required. This is necessary in cases where the splinkerette oligonucleotide ligated to genomic DNA regenerates the restriction site.

Note: We recommend first using **BstYI**. This enzyme is often sufficient for mapping most insertions. Longer DNA fragments can be isolated by using BglII or BamHI as needed.

When doing splinkerette PCR on a few samples, we also recommend performing splinkerette PCR on both the 5' and 3' ends of the transposable element. Each reaction should indicate the same genomic insertion site. If not, this is a good indicator that the fly line has multiple transposon insertions. Also, it is highly recommended to perform splinkerette PCR on a negative control genomic sample that does not contain P-elements, such as *white*^{1118} or Canton-S flies. This will verify that there are no contaminants in the reagents, as well as confirm that wild-type stocks are indeed free of P-elements.

Step 3. Ligation to Splinkerette Oligonucleotide

Table 3. Conditions for Ligating Digested Genomic DNA to Annealed Splinkerette Oligonucleotide

Components	Volume
Digested genomic DNA	35 µl
H_2O	2.5 µl
10X NEB Ligase Buffer	5 µl
annealed splinkerette oligonucleotide (see Table 14)	6 µl
NEB T4 DNA Ligase (400U/µl)	1.5 µl
Total	**50 µl**

3.1 Incubate at room temperature ≥ 2 hrs. Proceed directly to Round 1 PCR.
Note: ligation reactions can be incubated at 16ºC for 16 hrs, but an increase in efficacy was not found.

Step 4. Round 1 Splinkerette PCR

Table 4. Round 1 PCR Reaction.

Components	Volume
Ligated genomic DNA	10 µl
H_2O	8.25 µl
5x Phusion HF Buffer	5 µl
10 mm dNTP	0.5 µl
SPLNK#1,10 µM	0.5 µl
Primer #1, 10 µM (see Table 5)	0.5 µl
Phusion Taq (Finnzymes)	0.25 µl
Total	**25 µl**

Table 5. Primer Conditions for Round 1 PCR.

Purpose	Primer #1	xx anneal temp
3' end of any P-element	3'SPLNK#1	61ºC
5' end- CASPER	5'SPLNK#1-CASPR	61ºC
5' end- GAWB (GAL4 enhancer trap)	5'SPLNK#1-GAWB	63.5ºC
3'end – piggyBac	3'SPLNK-PB#1	58ºC
5'end – piggyBac	5'SPLNK-PB#1	64ºC

Table 6. PCR conditions for round 1 PCR.

98ºC 75 sec →	98ºC 20 sec 64ºC 15 sec (x 2) →	98ºC 20 sec xx ºC 15sec (x 30) → 72ºC 2 min	72ºC 7 min →	4ºC hold

4.1 Perform Round 1 PCR as detailed in Tables 4-6. We recommend using NEB Phusion Taq (Finnzymes) as this gave us the longest PCR fragments. PCR conditions have been optimized for Phusion Taq.

Step 5. Round 2 Splinkerette PCR

Table 7. Round 2 PCR Reaction.

Components	Volume
Round 1 PCR	≤1 µl
H_2O	17.25 µl
5x Phusion HF Buffer	5µl
10 mm dNTP	0.5 µl
SPLNK#2 ,10 µM	0.5 µl
Primer #2, 10 µM (see Table 8)	0.5 µl
Phusion Taq	0.25 µl
Total	**25 µl**

Table 8. Primer Conditions for Round 2 PCR.

Purpose	Primer #2	xx anneal temp
3'end of any P-element	3'SPLNK#2	58ºC
5' end- CASPER	5'SPLNK#2-CASPR	62ºC
5' end- GAWB (GAL4 enhancer trap)	5'SPLNK#2-GAWB	66ºC
3'end - piggyBac	3'SPLNK-PB#2	59ºC
5'end - piggyBac	5'SPLNK-PB#2	66ºC

Table 9. PCR Conditions for Round 2 PCR.

98ºC 75 sec →	98ºC 20 sec xx ºC 15 sec (x 30) → 72ºC 90 sec	72ºC 7 min →	4ºC hold

5.1 Perform Round 2 PCR as detailed in Tables 7-9. Best results are often obtained when using 0.5 µl round 1 PCR as template.

5.2 Analyze 5µl of Round 2 PCR on a 0.7% agarose gel. PCR band sizes will depend on which enzyme was used. Usually, BglII reactions yield ~1.5 kb bands, and BstYI reactions yield ~0.5 kb bands.

5.3 PCR bands can be gel extracted for sequence analysis. Alternatively, the remainder of the round 2 PCR reaction can be treated with Antarctic Phosphatase and Exonuclease and used directly for sequencing as detailed below. Good sequence runs can still be obtained after Step 6 even if only very weak bands are present on the gel.

Step 6. Antarctic Phosphatase/Exonuclease I treatment

Table 10. AntPho/ExoI Reaction Conditions.

Components	Volume
Round 2 splinkerette PCR	20 µl
10X NEB AP Buffer	3.0 µl
H_2O	3.0 µl
NEB Antarctic Phosphatase	2.0 µl
NEB Exonuclease I	2.0 µl
Total	**30 µl**

6.1 Incubate reactions at 37ºC for 2 hrs, followed by a 80ºC incubation for 15 min. Use 15µl of the reaction for sequencing with the appropriate sequencing primer listed in Table 11.

Table 11. Sequencing Primers

Purpose	Primer
3'end of any P-element	3'SPLNK-SEQ
5'end-CASPER	5'SPLNK-CASPR-SEQ
5'end-GAWB	5'SPLNK-GAWB-SEQ
3'end –piggyBac	3'SPLNK-PB-SEQ
5'end –piggyBac	5'SPLNK-PB-SEQ

Note: The following splinkerette specific sequence might be at the end of a sequence reaction:
GATCCCACTAGTGTCGACACCAGTCTCATTCAGCCACGGTCTCTCCTAGCAACGGTTACTCTTCG

Table 12. Restriction Enzymes with Compatible GATC Sticky Ends.

Name	Cut Site	Sites/Mb in *D. melanogaster* genome[1]	Average fragment length in base pairs[1]
BamH1	G↓GATCC	170.2	5874
BglII	A↓GATCT	183.3	5454
BstYI	R↓GATC Y	704.1	1420
BfuCI	↓GATC	2758.4	363

1. http://tools.neb.com/~vincze/gnsites/comp.php?genomes=Fruit_Fly

Table 13. Oligonucleotide Sequences

SPLNK-GATC-TOP	GATCCCACTAGTGTCGACACCAGTCTCTAATTTTTTTTTTCAAAAAAA
SPLNK-BOT	CGAAGAGTAACCGTTGCTAGGAGAGACCGTGGCTGAATGAGACTGGT GTCGACACTAGTGG
SPLNK#1	CGAAGAGTAACCGTTGCTAGGAGAGACC
SPLNK#2	GTGGCTGAATGAGACTGGTGTCGAC
3'SPLNK#1	CACTCAGACTCAATACGACAC
3'SPLNK#2	GGATGTCTCTTGCCGAC
3'SPLNK-SEQ	CGGGACCACCTTATG
5'SPLNK#1-CASPR	ATAGCACACTTCGGCACG
5'SPLNK#2-CASPR	ATTCGTCCGCACACAACC
5'SPLNK-CASPR-SEQ	CCTCTCAACAAGCAAACG
5'SPLNK#1-GAWB	TGGGAGAGTAGCGACACTCC

5'SPLNK#2-GAWB	GAGCTTTTTAAGTCGGCAAATATCG
5'SPLNK-GAWB-SEQ	CTCAACAAGCAAACGTGC
3'SPLNK-PB#1	GTTTGTTGAATTTATTATTAGTATGTAAG
3'SPLNK-PB#2	CGATAAAACACATGCGTC
3'SPLNK-PB-SEQ	ACGCATGATTATCTTTAAC
5'SPLNK-PB#1	ACCGCATTGACAAGCACG
5'SPLNK-PB#2	CTCCAAGCGGCGACTGAG
5'SPLNK-PB-SEQ	CGACTGAGATGTCCTAAATGC

Note: Splinkerette oligonucleotides SPLNK-GATC-TOP, SPLNK-BOT, SPLNK#1, SPLNK#2 are derived from [2].

Table 14. Reaction Conditions for Annealing Splinkerette Oligonucleotides

Component	Volume
SPLNK-BOT (150 ng/µl)	50 µl
SPLNK-GATC-TOP (150 ng/µl)	50 µl
10X NEB Buffer 2	100 µl
H_2O	800 µl
Total	1000 µl

14.1 Heat to 95ºC for 3 minutes. Allow to cool on bench to room temp (~30 mins). Store 200 µl aliquots at -20ºC.

Table 15. P-element Constructs Compatible with 3'SPLNK:

any pCaSpeR based construct	pGT1 (BG lines)	EPgy2 (EY lines)	P{SUPorP} (KG lines)
pGaWb (GAL4 enhancer traps)	pGatB/n	pPTGAL4	pPwl+hsGS (gene switch)
pUAST	pUASP	pUASdesFPc	EP
GS	Act5C>y+>GAL4 (Ay5C)	Flip-out constructs	ptubP-GAL80
UAS-CD8GFP	pWizDir	p[acman]	pRISE
pICOn	pTARG	p{Switch1}	p{Switch2}
pP{wHy}	pFRT	pP{wlo-inGS}	pP{wlo-hsinGS}
pPl25.1	*Drosophila* KP element	Dual-tagging gene trap vector pGT1	pUChsneo (neomycin)
pXP (Exelixis)	pP{neoFRT}	P{FRT(whs)}	Note: This list may not be complete.

Table 16. P-element Constructs Compatible with 5'SPLNK-CASPR:

pCaSpeR	P{SUPorP} (KG lines)	EPgy2 (EY lines)	pCa4B2G
pCa4B	Ganesh-Z1	pRISE	pUAST
pUASp	pXP (Exelixis)	piggyBac_PB (Exelixis)	pICON
p{Switch1}	p{Switch2}	pP{wHy}	RNAi cloning and transformation vector pFRiPE
Drosophila KP elements	Dual-tagging gene trap vector pGT1	pUChsneo (neomycin)	pP{neoFRT}
P{FRT(whs)}-PerrimonFRT	Note: This list may not be complete.		

5' and 3' piggyBac splinkerette PCR:
Primers were based on the pXL-Bac II backbone [3]. Derivates of this vector (*e.g.*, pXL-BacII-ECFP, pXL-Bac-DsRed, pXL-BacII-SAstopDsRed [1], pBAC-GH146 [4]) will work with these splinkerette primers. These primers are also compatible with the Exelixis piggyBac vector PB and its derivatives [5].

References:
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